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CIDEA interacts with liver X receptors in white fat cells

Agné Kulyté [a], Amanda T. Pettersson [a], Per Antonson [b], Britta M. Stenson [a], Dominique Langin [c,d,e], Jan-Åke Gustafsson [b], Bart Staels [f,g,h], Mikael Rydén [a], Peter Arner [a], Jurga Laurencikiene [a,*]

[a] Department of Medicine, Karolinska Institutet, Karolinska University Hospital, Huddinge, 141 86 Stockholm, Sweden
[b] Department of Biosciences and Nutrition, Karolinska Institutet, NOVUM, 141 83 Huddinge, Sweden
[c] Inserm, U858, Obesity Research Laboratory, Toulouse F-31432, France
[d] Université de Toulouse, UPS, Rangueil Institute of Molecular Medicine, Toulouse F-31432, France
[e] CHU de Toulouse, Biochemistry Laboratory, Biology Institute of Purpan, Toulouse F-31059, France
[f] Institut Pasteur de Lille, Lille F-59019, France
[g] Inserm, U1011, Lille F-59019, France
[h] Université Lille Nord de France, Lille F-59006, France

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ABSTRACT

Cell death-inducing DNA fragmentation factor alpha-like effector A (CIDEA) is endogenously expressed in human but not rodent white adipocytes. We performed a bioinformatic analysis of the human CIDEA sequence and found conserved amino-acid motifs involved in binding to nuclear receptors. Protein–protein binding experiments and transactivation assays confirmed that CIDEA binds to liver X receptors and regulates their activity in vitro. Cell fractionation demonstrated that CIDEA localizes to both the cytoplasm and the nucleus in human white adipocytes. The interaction between CIDEA and nuclear receptors could therefore be of importance for the regulation of metabolic processes in human adipose tissue.

Structured summary:
LXR-beta binds to **CIDEA** by pull down (View interaction)
TIF2 and **LXR-beta** physically interact by two hybrid (View interaction)
LXR-beta binds to **RAP250** by pull down (View interaction)
CIDEA and **LXR-beta** physically interact by two hybrid (View interaction)

1. Introduction

Cell death-inducing DNA fragmentation factor alpha-like effector A (CIDEA) is a protein of ~25 kDa that belongs evolutionarily to a family of cell death activators [1]. Although its exact functions are not clear, a number of recent reports have shown that CIDEA is involved in the regulation of lipid metabolism and energy expenditure. Ablation of CIDEA in mice causes a lean phenotype with increased energy expenditure and enhanced lipolysis [2]. In humans, CIDEA is highly and almost exclusively expressed in white fat cells [3,4]. Following diet-induced weight reduction, CIDEA was the most up-regulated gene in human subcutaneous adipose tissue [5]. A polymorphism in the human CIDEA gene, resulting in an amino acid substitution (V115F), is associated with protection against obesity [6]. Furthermore, low CIDEA expression in human adipocytes in vivo is associated with several features of the metabolic syndrome, such as increased waist circumference, accelerated basal (spontaneous) lipolysis and insulin resistance [4,7]. In addition, CIDEA knock-down in white human fat cells results in increased lipolysis [4]. Finally, CIDEA expression is upregulated in the fat cells of cancer cachexia patients and over-expression of CIDEA in fat cells inhibits glucose and stimulates fatty acid oxidation [8]. These human data suggest that CIDEA is involved in the regulation of fat mass in white fat cells.

However, the molecular mechanism of action of CIDEA and its intracellular localization are not clear and there appears to be important inter-cellular differences. Thus, in murine brown adipocytes, the green fluorescence protein (GFP)-mCIDEA fusion protein has been reported to localize to mitochondria [2]. In contrast, a hemagglutinin-tagged murine CIDEA was localized to the endo-

Abbreviations: CIDEA, cell death-inducing DNA fragmentation factor alpha-like effector A; DBD, DNA binding domain; GFP, green fluorescence protein; GST, glutathione *S*-transferase; LXR, liver X receptor; PPARγ, peroxisome proliferator-activated receptor γ

* Corresponding author. Address: Department of Medicine, Huddinge, Karolinska Institute, Novum, Hälsovägen 7, Level 4, D, SE-141 86 Stockholm, Sweden. Fax: +46 85855470.
E-mail address: Jurga.Laurencikiene@ki.se (J. Laurencikiene).

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plasmic reticulum in brown adipocytes and COS-7 cells [9]. A FLAG-tagged CIDEA stably over-expressed in MCF-7 cells shuttled between the nucleus and the cytoplasm [10]. In white adipocytes, an over-expressed CIDEA-fusion protein (CIDEA-GFP or CIDEA-V5) localized to lipid droplets [7,11]. Admittedly, it is difficult to conceive how CIDEA localized to the lipid droplet could affect the expression of enzymes involved in fatty acid metabolism [8].

The present study was undertaken to elucidate the intracellular localization of CIDEA in human white adipocytes and its possible interaction with other intracellular proteins.

2. Materials and methods

2.1. Cell culture

Human subcutaneous mature fat cells were obtained and purified from healthy subjects as described previously [12]. Isolated mature fat cells were used to obtain large amounts of adipocyte protein for immunoprecipitation studies. Isolation and differentiation of human adipocyte precursor cells from the stroma-vascular fraction was performed as described [13,14]. COS-7 cells and 3T3-L1 adipocytes (a murine fat cell line) were cultured and differentiated according to standard protocols.

2.2. Adenovirus infection and detection of cytosolic and nuclear proteins

Human CIDEA cDNA was cloned into pDUAL-CCM-EGFP adenoviral vector (VectorBiolabs, Philadelphia, PA) and adenovirus particles were produced by VectorBiolabs. Adenoviral infections were performed in human in vitro differentiated adipocytes as previously described [15]. The GFP-expressing adenovirus was used as control. Nuclei were isolated as previously described [16] with some modifications. Briefly, nuclei were isolated from 2×10^6 cells and suspended in 200 μl of nuclei lysis buffer (50 mM Tris–HCl, pH 7.5, 2.5 mM $MgCl_2$, 2% SDS, 2 mM DTT, 5 mM NaF, 1 mM Na_3VO_4, protease inhibitor cocktail Set V (Calbiochem, Gibbstown, NJ) and benzonase (final concentration 2.5 U/ml)). The lysates were incubated for 30 min on ice and then centrifuged at 15000×*g* for 20 min.

For SDS–PAGE, 40 μl of cytosolic or nuclear fraction, as well as 20 and 4 μl of nuclear fraction were loaded. Following primary antibodies were used: CIDEA (Sigma–Aldrich, St. Louis, MO), GAPDH, Lamin A/C (both from Cell Signaling Technology, Danvers, MA) and liver X receptor (LXR) (a gift from K.R. Steffensen, Karolinska Institute, Stockholm, Sweden).

2.3. Transactivation assay

COS-7 cell transfections were performed in serum free media for 2 h using the cationic lipid RPR120535B as described [17]. 3T3-L1 cells were transfected as described previously [8]. The plasmids used for transfections were the reporter pGal5TKpGL3 [17] and expression plasmids pGal4hLXRα, pGal4hLXRβ, pGal4-hPPARγDEF, pcDNA3.1 (Invitrogen, UK) and pcDNA3.1-hCIDEA [8]. pSV-βGal was used as a control of transfection efficiency and cell viability. The peroxisome proliferator-activated receptor γ (PPARγ) agonist BRL49653 (5 μM), the LXR agonists T0901317 and GW3965 (both at 1 μM) (Sigma–Aldrich) or vehicle alone were used to activate nuclear receptors after transfection. After 48 h cells were washed, lysed and luciferase activity was measured using the Dual-Luciferase™ Reporter Assay System (Promega, Madison, WI). Only experiments where the intra-experimental transfection efficiency and cell viability were comparable were used for the study. Each experimental group was tested in triplicate.

2.4. Glutathione S-transferase (GST) pull-down assay

GST and GST-human nuclear receptor fusion proteins were expressed in *Escherichia coli* BL21(DE3)-pLysS from pGEX-based plasmids and purified as described [18]. CIDEA and RAP250 [19] were transcribed in vitro and translated with ^{35}S-methionine using TNT coupled reticulocyte lysate system (Promega). GST bound fusion proteins were incubated for 2 h at 4 °C with 2 μl of ^{35}S-methionine-labeled protein in the presence of the 1 μM T0901317 or with vehicle alone in 200 μl of incubation buffer (50 mM KPi, pH 7.4, 10% (v/v) glycerol, 100 mM NaCl, 1 mM $MgCl_2$, 0.1% Tween) supplemented with 1.5 mg/ml bovine serum albumin. Precipitated proteins were detected as described [18].

2.5. Mammalian two-hybrid assay

We have used an assay based on the Matchmaker™ Mammalian Two-Hybrid system (Clontech, Mountain View, CA), which allows testing protein–protein interactions in transfected mammalian cells. The following plasmids were used for the transfections; pUAS-tk-Luc (luciferase reporter), pM (coding for DNA binding domain (DBD)), pVP16 (coding for activation domain), pM-CIDEA, pM-TIF2, pVP16-LXRβ, pCMVβ-gal (to correct for transfection efficiency) [19]. Transient transfections were performed in fat cells differentiated in vitro for 10–12 days using Lipofectamine/Plus reagent (Invitrogen). Cells were lysed 24 h after transfection and assayed for luciferase and β-galactosidase activities. Each experimental group was tested in triplicate.

2.6. Immunoprecipitation experiments

Mature human adipocytes were lysed in a buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.25% sodium deoxycholate and 1% NP-40) supplemented with protease inhibitors. The pcDNA3.1-hCIDEA and Gal4-DBD constructs were translated in vitro using TNT coupled reticulocyte lysate system (Promega) and ^{35}S-methionine (GE Healthcare, UK). The amount and integrity of the respective proteins were assessed by running a small aliquot on SDS–PAGE and analyzing the gel by autoradiography. After pre-clearing with Protein-A sepharose (GE Healthcare), a similar amount of in vitro translated proteins was incubated with 400 μg of protein lysate from human mature adipocytes for 1 h. A mixture of monoclonal anti-LXRα/NR1H3 and anti-LXRβ/NR1H2 antibodies (R&D Systems, Minneapolis, MN) or mouse serum IgG were added and lysates were incubated overnight under rotation. Complexes were precipitated with Protein A-sepharose, washed and eluted by boiling in SDS-sample buffer. Samples were then fractionated by SDS–PAGE followed by autoradiography.

2.7. Statistical analysis

Statistical significance was determined using non-parametric Mann–Whitney test. $P < 0.05$ was considered significant. Data are mean ± standard deviation (S.D.).

3. Results

3.1. The CIDEA sequence reveals motifs for nuclear receptor binding

In an effort to better understand the molecular mechanisms through which CIDEA mediates its effects, an analysis of the CIDEA protein sequence was performed to search for possible protein–protein interaction domains. Two motifs were found that could mediate interaction with nuclear receptors [20]. In the

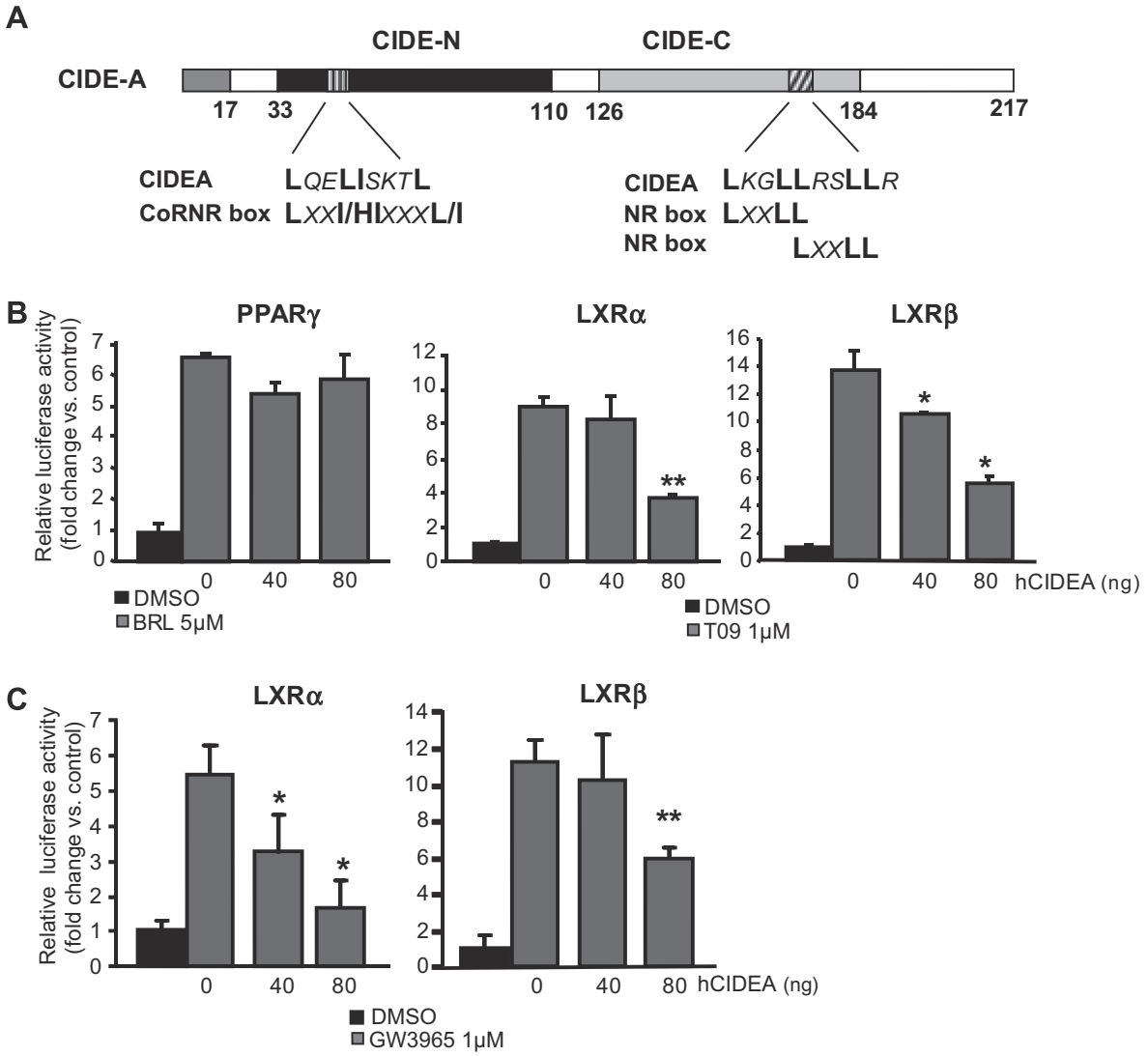

Fig. 1. CIDEA regulates activity of nuclear receptors. (A) Analysis of CIDEA protein sequence showing motives for nuclear receptor binding. (B) Transactivation assays in COS-7 cells ($n = 4$). (C) Transactivation assays in 3T3-L1 cells ($n = 3$). Values show the effect of agonist in each condition and are expressed as mean ± S.D., $^{*}P < 0.05$, $^{**}P < 0.01$.

C-terminal domain there are two overlapping nuclear receptor (NR)-boxes while the N-terminal domain contains a CoRNR box, which is present in co-repressors of nuclear receptors (Fig. 1A).

3.2. CIDEA regulates activity of LXRs

We hypothesized that CIDEA might regulate the activity of nuclear receptors involved in adipocyte metabolism or differentiation. Therefore, COS-7 cells were co-transfected with vector containing cDNA for PPARγ fused to Gal4-DBD, a Gal4-responsive reporter and different amounts of hCIDEA-expressing vector. Cells were treated with the PPARγ agonist BRL49653. Our data showed that presence of CIDEA had no effect on PPARγ activity in this system (Fig. 1B). We therefore chose to investigate CIDEAs effect on the nuclear receptor LXR which has been implicated in the regulation of fat cell metabolism [21–23]. Both LXRα and LXRβ markedly up-regulated luciferase activity following addition of the LXR agonist T0901317 (Fig. 1B). Co-transfection of CIDEA resulted in a concentration-dependent decrease of both LXRα and LXRβ activities. CIDEA over-expression had no effect on luciferase activity in the absence of T0901317 (data not shown). These effects were not restricted to COS-7 cells as similar effects were observed in murine 3T3-L1 cells (Fig. 1C).

3.3. CIDEA binds to LXR in vivo and in vitro

To investigate whether CIDEA can bind directly to LXR, a pull-down assay was performed using an in vitro translated ^{35}S-Cys-labeled human CIDEA and GST-tagged LXRβ. In line with the data from the transactivation assays, binding was observed between CIDEA and LXR in this non-cellular assay (Fig. 2A). CIDEA binding to LXR was not affected by LXR agonist T0901317. As a positive control for the analysis, we used an in vitro translated fragment containing the NR-box1 from RAP250 [19], which showed a ligand-enhanced binding to LXRβ (Fig. 2A). We also determined whether CIDEA and LXR interact in adipocyte cell extracts by co-immunoprecipitation. To this end, CIDEA was in vitro-translated in the presence of ^{35}S-methionine and incubated with adipocyte lysates in presence of anti-LXR or control IgG antibodies. The Gal4-DBD has no reported binding to LXR and was therefore chosen as a negative control. Eluted proteins were analyzed by SDS–PAGE and autoradiography. A clear band corresponding to the size of CIDEA was detected in the samples immunoprecipitated with the anti-LXR antibody but not with unspecific IgG antibodies (Fig. 2B). There was no detectable band in samples containing ^{35}S-methionine labeled Gal4-DBD immunoprecipitated with anti-LXR.

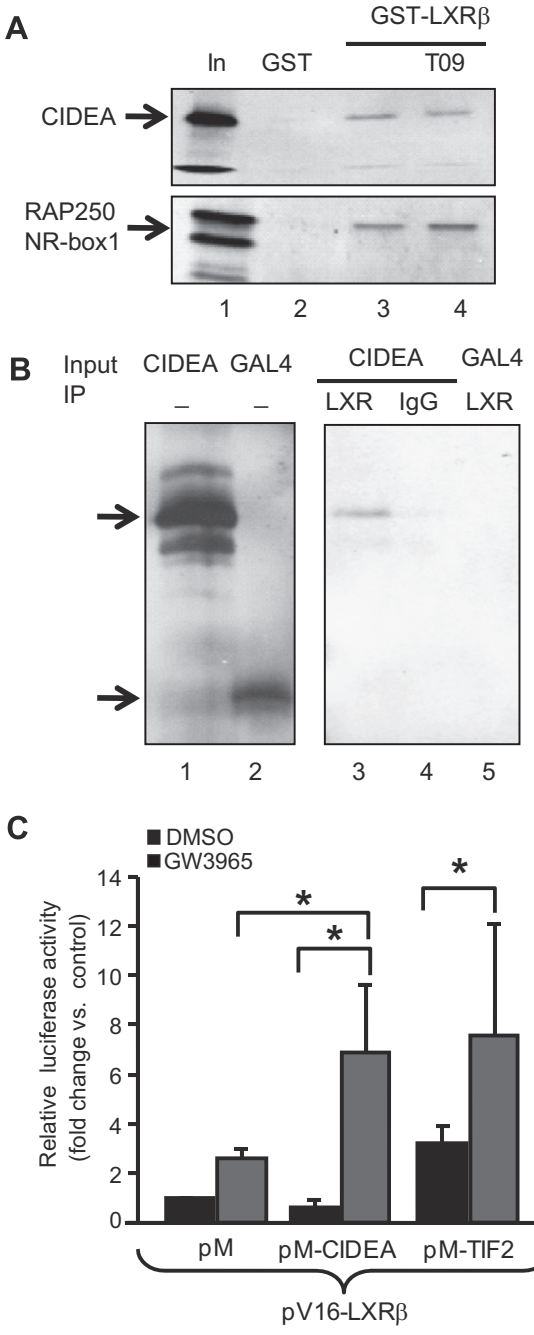

Fig. 2. CIDEA binds to LXR in vitro and in vivo. (A) GST pull-down assay was performed with in vitro translated CIDEA and GST-coupled nuclear receptors. Input (lane 1), GST alone (lane 2), GST-LXRβ (lanes 3, 4) were used to precipitate CIDEA. LXR agonist T0901317 (T09) (lane 4) was used. RAP250 NR-box1 served as a positive control. (B) Co-immunoprecipitation of ^{35}S-CIDEA or ^{35}S-Gal4-DBD with LXR. Lanes 1 and 2 show input samples, lanes 3–5 show immunoprecipitated samples. (C) Adipocytes differentiated in culture were transfected with the empty pM, pM-CIDEA or pM-TIF2 vectors together with pVP16-LXRβ and luciferase reporter as described in Section 2. Results represent the mean value of triplicate ± S.D. from one representative experiment $n = 3$, $^{*}P < 0.05$.

To further demonstrate an interaction between CIDEA and LXR in a living cell system, a mammalian two-hybrid assay [19] was set up in differentiated human adipocytes. Co-transfection of the cells with pM-CIDEA and pVP16-LXRβ constructs resulted in a high ligand-dependent activity of the reporter gene (Fig. 2C), suggesting an interaction between CIDEA and LXRβ in human fat cells. In similar experiments, there was no interaction between CIDEA and PPARγ (figure not shown). TIF2, a well established co-activator of nuclear receptors served as a positive control [24].

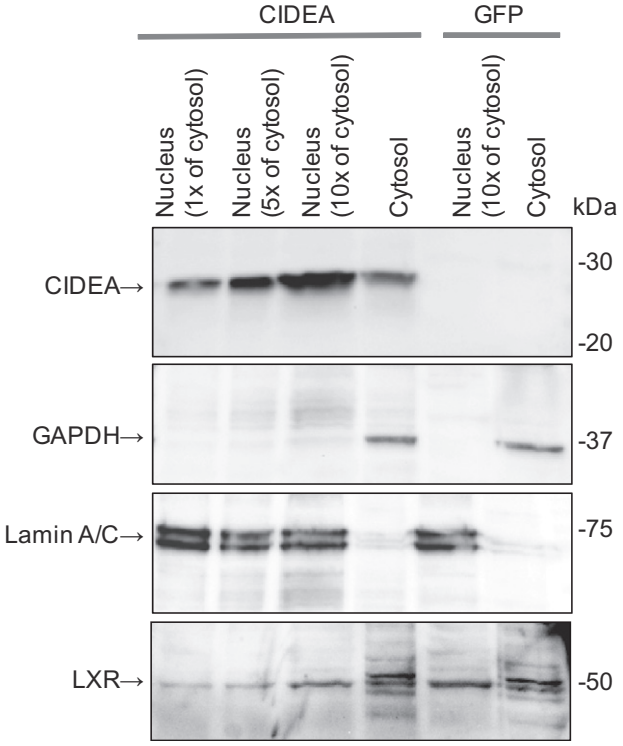

Fig. 3. CIDEA is localized to nucleus and cytoplasm in adipocytes. Western blot detection of CIDEA, GAPDH, Lamin A/C and LXR in cytosolic and nuclear extracts from human adipocytes infected by CIDEA or GFP.

3.4. CIDEA is localized to both cytoplasm and the nucleus in adipocytes

Due to the low levels of endogenous CIDEA expressed in in vitro differentiated human adipocytes, adenoviral infections were performed with CIDEA- or GFP-expressing adenoviral particles followed by extraction of nuclear and cytoplasmic lysates. Western blot analysis demonstrated that virally over-expressed CIDEA and endogenous LXR localized to both the cytoplasmic and the nuclear fractions to a similar extent (Fig. 3). The nuclear protein Lamin A/C and the cytoplasmic protein GAPDH were used as controls for purity of the nuclear and cytoplasmic fractions, respectively.

4. Discussion

Recent studies in humans and rodents suggest an important role for CIDEA in regulating the metabolism of white adipocytes, but the molecular mechanisms have remained largely unclear. Here we provide evidence for an additional and hitherto unknown mode of action for CIDEA in white fat cells.

Based on the analysis of the CIDEA protein sequence, we hypothesized that it could interact with nuclear receptors. Given the fact that CIDEA is predominantly expressed in adipocytes, we focused on the possible interaction with nuclear receptors with established roles in fat cell metabolism, namely PPARγ and LXR [25,26]. We demonstrate a physical interaction between CIDEA and LXR using a set of distinct techniques including transactivation and mammalian two-hybrid experiments. The latter suggest that the CIDEA/LXR interaction is taking place in living cells and is not solely an in vitro phenomenon.

The transactivation data obtained in COS-7 and murine 3T3-L1 cells indicate that CIDEA attenuates LXR activity. Thus, CIDEA may act as a co-repressor of LXR, which is in agreement with the finding that CIDEA binds to LXR in a ligand-independent manner. Although CIDEA posses NR-binding motifs, mutations of NR- and

CoRNR-boxes did not interfere with CIDEA binding to LXR in GST pull-down assays or CIDEA co-repressor activity in transactivation assays (data not shown). Therefore, it is likely that CIDEA interacts with LXR via different domain than known NR-binding motifs. It is at present unclear whether additional proteins bind or interact with the CIDEA/LXR complex. If so, it is quite likely that such regulatory proteins are differentially expressed in different cell types. In addition, CIDEA is almost exclusively expressed in fat cells [2–4], which could confer tissue-selective effects to LXRs. CIDEA has been reported to induce ubiquitination and degradation of AMP-activated protein kinase [9]. The amount of LXR is not affected by CIDEA over-expression (Fig. 3), suggesting that CIDEA does not affect stability of this nuclear receptor. At present, we do not know whether CIDEA affects the recruitment of LXR to DNA or its transcriptional activity.

Studies on the intracellular localization of CIDEA are hampered by the absence of specific antibodies. Different molecular tags and cell systems have therefore been used in order to elucidate the intracellular compartmentalization of CIDEA [7,9–11]. Different CIDEA-fusion proteins localize to several compartments/organelles in different cells. In our fractionation studies we over-expressed untagged CIDEA. Although we admit that over expression might cause some artificial effects, our data indicate that CIDEA is localized to both the nucleus and the cytoplasm in human adipocytes. A previous report demonstrated that nuclear over-expression of CIDEA induced apoptosis in MCF-7 cells [10]. In contrast, we did not observe any increase in apoptosis or necrosis following CIDEA over-expression in our cell systems (data not shown).

It would be important to determine, which metabolic pathways are affected by CIDEA/LXR interaction. Both CIDEA and LXR regulate adipocyte lipolysis and substrate oxidation [3,4,8,21,27]. On the other hand, LXR is a multifunctional transcription factor and we cannot exclude that CIDEA would affect other metabolic pathways, regulated by LXR in adipocytes. Additional studies using more sophisticated in vivo experimental systems are needed to establish a physiological role for the CIDEA/LXR crosstalk.

In conclusion, both LXR and CIDEA regulate metabolism in white adipocytes. We propose that CIDEA is a regulator of LXR and possibly other nuclear receptors in fat cells. These interactions could be of importance for adipose tissue metabolism and obesity, an issue that will be addressed in future studies.

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